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(54) Title: USE OF CYCLOLIGNANS AS INHIBITORS OF IGF-1 RECEPTOR FOR TREATMENT OF MALIGNAT DISEASES

(57) Abstract: The invention refers to the use of certain cyclolignans as specific inhibitors of the insulin-like growth factor-1 receptor for treatment of the malignant diseases neuroblastoma, myeloma, glioblastoma and lung cancer, as well as benign tumours. Preferred compounds are picropodophyllin and deoxypodophyllotoxin.

NEW USE

The present invention refers to the use of certain cyclolignans as specific inhibitors of the insulin-like growth factor-1 receptor, the IGF-1R, for treatment of the malignant diseases neuroblastoma, myeloma, glioblastoma and lung cancer, as well as benign tumours.

BACKGROUND OF THE INVENTION

The insulin-like growth factor-1 receptor plays an important role in proliferation, protection against apoptosis and transformation of malignant cells. The IGF-1R is also important for maintaining the malignant phenotype of tumour cells, and is involved in tumour cell protection against anti-tumour therapy. In contrast, the IGF-1R does not seem to be an absolute requirement for normal cell growth.

Dysregulation of IGF-1 signaling can also contribute to the neoplastic growth of benign tumours like uterine leiomyomas as described by Burroughs, K. D., *et al.* in J. *Endocrinology* 2002, Vol.172, 83-93 and Druckmann, R. and Rohr, U. D. in *Maturitas* 41, Suppl. 1, 2002, S65-S83.

The IGF-1R consists of two identical extracellular α-subunits that are responsible for ligand binding, and two identical β-subunits with a transmembrane domain and an intracellular tyrosine kinase domain. The ligand-receptor interaction results in phosphorylation of tyrosine residues in the tyrosine kinase domain, which spans from amino acid 973 to 1229, of the β-subunit. The major sites for phosphorylation are the clustered tyrosines at position 1131, 1135 and 1136 (LeRoith, D., *et al.*, *Endocr Rev* 1995 April; 16(2), 143-63). After autophosphorylation the receptor kinase phosphorylates intracellular proteins, like insulin receptor substrate-1 and Shc, which activate the phosphatidyl inositol-3

kinase and the mitogen-activated protein kinase signaling pathways, respectively.

Based on the pivotal role of IGF-1R in malignant cells, it becomes more and more evident that IGF-1R is a target for cancer therapy (Baserga, R., *et al.*, *Endocrine* Vol. 7, no. 1, 99-102, August 1997). One strategy to interfere with IGF-1R activity is to induce selective inhibition of the IGF-1R tyrosine kinase.

The IGF-1R is a member of the tyrosine kinase receptor family, which also includes the receptors of insulin, epidermal growth factor (EGF), nerve growth factor (NGF), and platelet-derived growth factor (PDGF). A number of synthetic tyrosine kinase inhibitors, called tyrphostins, have been studied by Párrizas, M., *et al.*, *Endocrinology* 1997, Vol. 138, No. 4, 1427-1433. The major and very serious disadvantage with all tyrphostins active on IGF-1R is that they cross-react with the insulin receptor, since these receptors are highly homologous. However, some of the tyrphostins showed a moderate preference for IGF-1R, suggesting that it could be possible to design and synthesize small molecules capable of discriminating between these two receptors. Substrate competitive inhibitors of the IGF-1 receptor kinase, which however cross-react with the insulin receptor, are discussed by Blum, G., *et al.* in *Biochemistry* 2000, 39, 15705-15712 and in *J. Biol. Chem.*, 2003, 278, 40442-40454.

Drugs containing the notoriously toxic cyclolignan podophyllotoxin have been used for centuries, and its anti-cancer properties have attracted particular interest. Undesired and severe side effects of podophyllotoxin have, however, prevented its use as an anti-cancer drug. The mechanism for the cytotoxicity of podophyllotoxin has been attributed to its binding to β-tubulin, leading to inhibition of microtubule assembly and mitotic arrest. The *trans* conformation in the lactone ring of podophyllotoxin has been shown to be required for binding to β-tubulin. In contrast, its stereoisomer picropodophyllin, which has a *cis* configuration in

the lactone ring, has a 50-fold lower inhibitory effect on microtubule polymerisation and a more than 35-fold higher LD_{50} value in rats. Because of the negligible anti-microtubule effect of picropodophyllin this compound has attracted little interest.

During the last decades the major interest in podophyllotoxin derivatives has concerned etoposide, which is an ethylidene glucoside derivative of 4´-demethyl-epipodophyllotoxin. Etoposide, which has no effect on microtubules (or on the IGF-1R), is a DNA topoisomerase II inhibitor, and is currently being used as such in cancer therapy.

The specific cancer type myeloma and certain forms of neuroblastoma, glioblastoma and lung cancer respond poorly to treatment and have today a miserable clinical outcome. There are also several benign neoplastic diseases associated with a reduced quality of life, which today are without efficient treatment.

PRIOR ART

PCT/SE02/01202 discloses the use of specific cyclolignans, wherein the carbon atoms in positions 9 and 9´ have *cis* configuration, for specific inhibition of the insulin-like growth factor-1 receptor. Said compounds can be used for treatment of IGF-1R dependent diseases, such as cancer, psoriasis, arteriosclerosis and acromegaly. A preferred compound is picropodophyllin.

PCT/SE02/01223 discloses new compounds and the use thereof, as well as the use of cyclolignans having a *trans* configuration of the lactone ring, as specific inhibitors of the insulin-like growth factor-1 receptor. Said compounds can be used for treatment of IGF-1R dependent diseases, especially cancer. Especially deoxypodophyllotoxin can be used for treatment of leukemia in humans.

The Chemistry of Podophyllum by J.L. Hartwell *et al.*, Fortschritte der Chemie organischer Naturstoffe 15, 1958, 83-166, gives an overview of podophyllotoxin and different derivatives

thereof, commercially derived from two species of plants, *Podophyllum peltatum* and *Podophyllum emodi*.

Although some cyclolignans, such as deoxypodophyllotoxin, have been noted to possess cytotoxic activity, their activity has never been associated to an inhibition of IGF-1R. In fact, their mechanism of action has not been characterized or has just been believed to be a binding to microtubuli like that of podophyllotoxin and therefore they have been expected to be of limited usefulness.

Picropodophyllin, on the other hand, has generally been considered to lack biological activity as stated by Ayres, D.C., and Loike, J.D., Lignans. Chemical, biological and clinical properties. Cambridge University Press, Cambridge, 1990.

OBJECTS OF THE INVENTION

The object of the invention is to find new methods for treatment of neuroblastoma, myeloma, glioblastoma and lung cancer by means of a specific inhibition of the tyrosine kinase of the insulin-like growth factor-1 receptor. Another object of the invention is to find new treatments of benign tumours.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a computer model of a 12 amino acid peptide comprising the tyrosines 1131, 1135 an 1136 of the IGF-1 receptor.

Figure 2 shows the structural formulas of the compounds picropodophyllin and deoxypodophyllotoxin.

DESCRIPTION OF THE INVENTION

The three-dimensional structure of a 12-amino acid sequence of the IGF-1R tyrosine domain, including the tyrosine residues at position 1131, 1135 an 1136, was analysed using a computer programme in order to find compounds having the ability to mimic the tyrosine residues and interfere with the phosphorylation

thereof. It was then discovered, when using a 12-amino acid peptide, that two of the three key tyrosines, that is 1135 and 1136, which have to be autophosphorylated in IGF-1R for activation, could be situated as close as 0.95 nm (9.5 Å) from each other, and that the apparent angle between these groups was about 60°. The configuration of said sequence is shown in Figure 1. Such a short distance has not been observed for the corresponding tyrosines in the insulin receptor.

Molecular modelling showed that an inhibitory, mimicking molecule could consist of two benzene rings separated by only one carbon atom. When a two-carbon bridge was tried, the distance between the substituents of the benzene rings was too long, i.e. about 1.3 nm (13 Å).

The substituents corresponding to the hydroxy groups in the tyrosines were selected to be methoxy or methylenedioxy groups, since they are chemically relatively stable, i.e. they are not oxidized or phosphorylated. The distance between these substituents seems to be about 0.95 ± 0.10 nm (9.5 ± 1.0 Å).

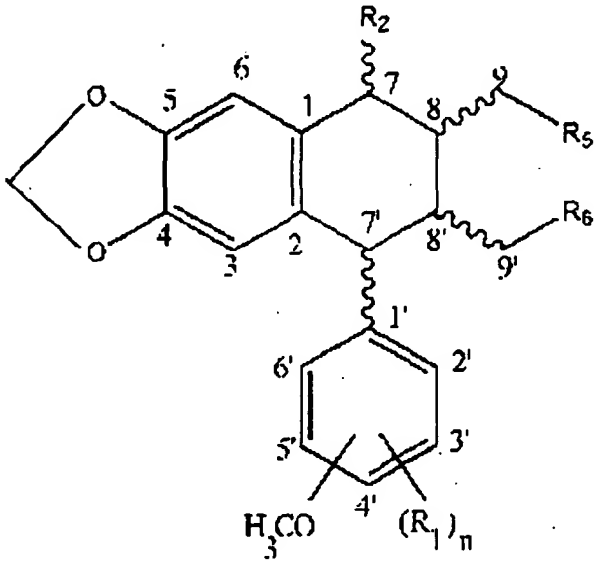
It was then surprisingly found that two angled and substituted benzene rings of the cyclolignans podophyllotoxin, deoxypodophyllotoxin and picropodophyllin could structurally almost exactly mimic the tyrosines 1135 and 1136, indicating that said compounds may fit into the tyrosine kinase pocket and thereby interfere with the autophosphorylation of the tyrosine residues. Figure 1 also shows the space structure of picropodophyllin and deoxypodophyllotoxin. Thus, in contrast to the effect on microtubuli, the IGF-1R inhibition was not limited to cyclolignans with a *trans* configuration in the lactone ring.

In order to penetrate the receptor, an inhibitory molecule has to be small. When for instance podophyllotoxin was conjugated with a glucoside derivative, podophyllotoxin-4,6-O-benzylidene-β-D-glucopyranoside, the effect on IGF-1R completely disappeared. Furthermore, following reduction of the lactone ring to a diol

structure, the size of the molecule increased due to the reduced substituents sticking out from the molecule, resulting in a dramatically reduced activity of the compounds. Increasing the size by forming methylenedioxy derivatives or acetonides of podophyllotoxindiol also resulted in compounds with little or no activity.

The inhibitor molecule also has to be relatively non-polar, so that it can freely penetrate cell membranes and the IGF-1 receptor, but sufficiently polar to be reasonably soluble in water. The polarity of the molecule is determined by the number and nature of the oxygen functions. The polarity seems to be optimal when the water solubility is between that of deoxypodophyllotoxin, i.e. about 0.01 mM, and that of podophyllotoxin, about 0.1-0.2 mM. No charged or highly polar groups should be present in the molecule.

The invention refers to the use of a compound of the formula I



I

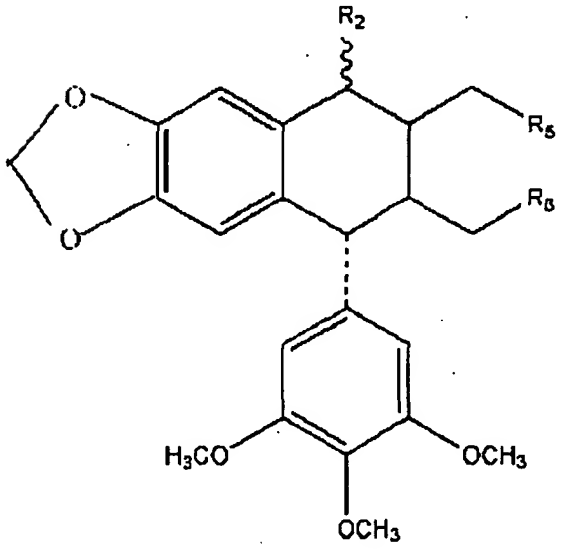
wherein R_1, which can be the same or different, is OH or OCH_3, n is 0, 1 or 2, R_2 is H, OH, O, NOH, $NOCH_3$, OCH_3, OCOH, OCH_2CH_3, or $OCOCH_3$, and R_5 and R_6, which can be the same or different, are H, OH, O, OCH_3, $OOCH_3$, OCOCH, OCH_2CH_3, $OOCH_2CH_3$, or $OCOCH_3$, or together an ether or a lactone, and which optionally contains a double bond $\Delta^{7(8)}$ or $\Delta^{8(8')}$, as a specific inhibitor of the insulin-like growth

factor-1 receptor for the preparation of a medicament for treatment of neuroblastoma, myeloma, glioblastoma and lung cancer.

The invention also refers to the use of a compound of the formula I, as defined above, for the preparation of a medicament for treatment of benign tumours. Examples of benign neoplastic diseases are neurofibromatosis, fibromatosis, lipomatosis, papillomatosis and meningeomas.

According to a special aspect the invention refers to the use of compounds of the formula I for the preparation of a medicament for treatment of the benign tumour leiomyoma. Uterine leiomyoma is the most frequently occurring tumour of the female reproductive tract and clinically affect at least 25 % of women in their fertile age. It causes a number of problems, such as infertility, lost pregnancy, pain and menorrhagia, and is the primary cause of hysterectomies in women.

The invention especially refers to the use of compounds of the formula II



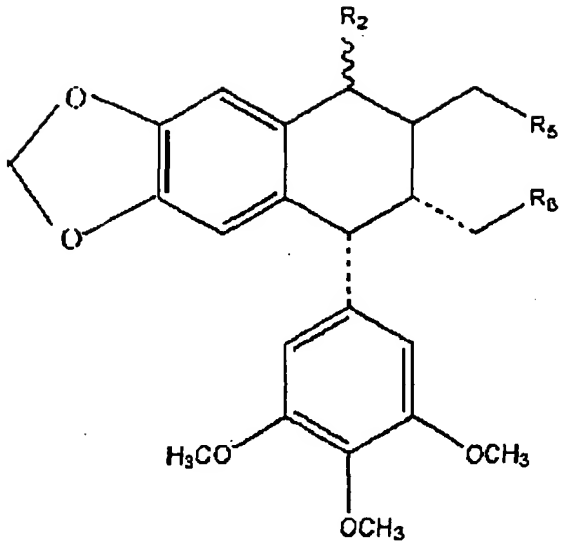
II

wherein R_2 is H, OH, O, NOH, $NOCH_3$, OCH_3, OCOH, OCH_2CH_3, $OCOCH_3$, and R_5 and R_6, which can be the same or different, are H, OH, O, OCH_3, $OOCH_3$, OCOCH, OCH_2CH_3, $OOCH_2CH_3$, or $OCOCH_3$, or together an ether or a lactone, and which optionally contains a double bond $\Delta^{7(8)}$ or $\Delta^{8(8')}$, as a specific inhibitor of the insulin-like growth factor-1 receptor for the preparation of a medicament for treatment of neuroblastoma, myeloma, glioblastoma and lung cancer, or for treatment of benign tumours.

Notably, the carbons in positions 9 and 9' of all the compounds of the formula II have a *cis* configuration, i.e. the 8-9 and 8'-9' bonds are located in or above the plane of the carbon ring (beta bonds), as indicated by the solid lines in the formula II. A wavy line, as between the carbons 1' and 7' in the formula I, indicates that the bond can be either an alpha or a beta bond. An alpha bond, that is below the plane of the carbon ring, is illustrated by a dashed line. The phenyl group should preferably be in α-position, as is demonstrated by picropodophyllin.

Preferred *cis*-compounds to be used according to the invention can be selected from the group consisting of picropodophyllin, deoxypicropodophyllin, beta-apopicropodophyllin and alpha-apopicropodophyllin.

The invention also refers to the use of a compound having the formula III



III

wherein R_2 is H, OH, O, NOH, $NOCH_3$, OCH_3, OCOH, OCH_2CH_3, $OCOCH_3$, and R_5 and R_6, which can be the same or different, are H, OH, O, OCH_3, $OOCH_3$, OCOCH, OCH_2CH_3, $OOCH_2CH_3$, or $OCOCH_3$, or together an ether or a lactone, and which optionally contains a double bond $\Delta^{7(8)}$, as a specific inhibitor of the insulin-like growth factor-1 receptor for the preparation of a medicament for treatment of neuroblastoma, myeloma, glioblastoma and lung cancer, or for treatment of benign tumours.

Notably, the carbons in positions 9 and 9´ of all the compounds of the formula III have a *trans* configuration. i.e. the 8-9 bond is located in or above the plane of the carbon ring (beta bond) and the 8´-9´ bond is located below the plane, as indicated by the solid and dashed line in the formula III, respectively. The phenyl group should preferably be in α-position, as is demonstrated by deoxypodophyllotoxin.

Preferred *trans*-compounds to be used according to the invention can be selected from a group consisting of deoxypodophyllotoxin, epipodophyllotoxin, and acetylpodophyllotoxin.

Especially preferred compounds are picropodophyllin or deoxypodophyllotoxin. The chemical structures of said compounds are shown in Figure 2.

Podophyllotoxin and deoxypodophyllotoxin, used as starting material for the synthesis of the described picro derivatives, are naturally occurring in plants. For the preparation of said substances in pure form, dried and finely ground rhizomes of e.g. *Podophyllum emodi* or *Podophyllum peltatum* are extracted with organic solvents. The extract is then filtered and concentrated on silica gel. The fractions containing the substances are collected and the latter are further purified by chromatography on acid alumina and silica gel etc., and finally recrystallized.

Deoxypicropodophyllin and picropodophyllin can be prepared from deoxypodophyllotoxin and podophyllotoxin, respectively. One mg of the latter was dissolved in 70 % aqueous methanol. To the solution was added 20 mg of sodium acetate and the mixture was then incubated for 20 h at 55°C. After evaporation of the alcohol, the product was extracted with ethyl acetate, and then purified by chromatography on silica gel, mobile phase: hexane-ethyl acetate mixtures, and/or octadecylsilane-bonded silica, mobile phase: aqueous methanol.

Alpha- and beta-apopicropodophyllin can be prepared from podophyllotoxin by incubation in a buffered ethanolic solution at elevated temperature, as described by Buchardt, O. *et al.*, *J Pharmaceut Sci* 75, 1076-1080, 1986. The total synthesis of picropodophyllin and its apo derivatives have been described by Gensler, J.W., *et al.*, *J Am Chem Soc* 82, 1714-1727, 1960.

As additional examples of compounds of the formula I can be mentioned: epipicropodophyllin, picropodophyllone, 4´-demethyl-picro-podophyllin, and the acetate derivative of picropodophyllin

and the methyl ester and ethyl ester derivatives of picropodophyllic acid.

In case of tumours not completely dependent on IGF-1R, the compounds of the invention can be useful to potentiate the effects of other anti-tumour therapies. The invention therefore also refers to the use of a compound of the formula I in combination with another cytostaticum. As examples of cytostatica which can be used together with a cyclolignan of the invention can be mentioned vincristin, taxol and etoposide.

For parenteral administration, the compounds may be administered as injectable dosages or by continuous intravenous infusion of a solution, suspension or emulsion of the compound in a physiologically acceptable diluent as the pharmaceutical carrier, which can be a sterile liquid, such as water, alcohols, oils, emulsions, and other acceptable organic solvents, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants.

The compounds can also be administered in the form of a depot injection or implant preparation, which may be formulated in such a manner as to permit a sustained release of the active ingredient.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, troches, powders, solutions, suspensions or emulsions.

For topical application the compounds can be administered in the form of an unguent, cream, ointment, lotion or a patch.

The results of our biological experiments show that relatively low concentrations of the IGF-1R inhibitors can be sufficient to cause tumour cell death. However, it is believed that it is important to keep a sufficient and relatively constant plasma concentration of the inhibitors over lengthy periods, to allow the inhibitors to continuously saturate and block all IGF-1Rs, and in this way eventually kill all of the malignant cells. Therefore, continuous infusion of the compounds of the invention, in

connection with monitoring the plasma concentration, may be the strategy of treatment instead of giving one single or repetitive injections with relatively long time intervals, such as once daily or weekly, which may lead to repeated reactivation of IGF-1R between treatments.

The invention consequently also refers to a method of treatment of the particularly malignant tumours neuroblastoma, myeloma, glioblastoma and lung cancer in a mammal, comprising the steps of administrating a pharmaceutical composition, containing a compound having the formula I in combination with a physiologically acceptable carrier, by constant infusion to a patient suffering from a tumour, monitoring the plasma level of the compound, and adjusting the rate of infusion to keep the plasma level at a concentration of 0.05-5.0 μM, for a period of time being sufficient for the tumour to be retarded or to disappear.

The invention also refers to a method of treatment of benign tumours in a mammal, comprising the steps of administrating a pharmaceutical composition, containing a compound having the formula I in combination with a physiologically acceptable carrier, by constant infusion to a patient suffering from a tumour, monitoring the plasma level of the compound, and adjusting the rate of infusion to keep the plasma level at a concentration of 0.05-5.0 μM, for a period of time being sufficient for the tumour to be retarded or to disappear.

EXPERIMENTAL

Materials

Chemicals

Cell culture reagents that are media, fetal calf serum and antibiotics, were purchased from Gibco, Sweden. All other chemicals unless otherwise stated were from Sigma (St. Louis. MO, USA). A mouse monoclonal antibody against phosphotyrosine (PY99) and a polyclonal antibody against α-subunit of IGF-1R (N20) were obtained

from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). A monoclonal antibody against the α-subunit of IGF-1R (αIR-3) was purchased from Oncogene Science (N.Y., USA). Picropodophyllin (99.97 % purity) and deoxypodophyllotoxin (99.97 % purity) were obtained as gifts from Analytecon SA, Pre Jorat, Switzerland. Solvents were of analytical reagent grade and Sep-Pak C_{18} cartridges were from Waters Associates (Milford, MA, USA).

Cell cultures

Human lung cancer cells (A549) were maintained in Ham's F12K medium with 2 mM L-glutamine and 10% fetal bovine serum. The human myeloma cell lines EJM, Karpas and RPMI 8226 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and with 2 mM glutamine, 1 % benzyl-penicillin and streptomycin. The human neuroblastoma cell line SH-SY5Y and glioblastoma cells (U-87MG) were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and with 2 mM glutamine, 1 % benzyl-penicillin and streptomycin. The cells were grown in tissue culture flasks maintained at 95 % air/5% CO_2 atmosphere at 37°C in a humidified incubator. For the experiments 25,000 cells were cultured in 96-well plastic plates.

Human uterine leiomyoma cells were isolated and cultured from surgical specimen essentially as described previously for vascular smooth muscle cells (Ross R., J. Cell Biol. 50: 172-186, 1971). Briefly, the cells were allowed to migrate from the primary explants and were subsequently passaged at confluence. Cells were maintained in F12 medium containing 15 % fetal bovine serum, 0.05 mg/ml ascorbic acid, 2 µg/ml fungizone and 200 IU/ml of penicillin. Cultured cells were a uniform population of human leiomyoma cells identified both by their morphology and by immunostaining. Culturing was performed at a temperature of 37°C, humidity of 85 % and at a CO_2 concentration of 5 % in air. The medium was changed twice a week and the cells were harvested at passage 2 to 8 using a solution of trypsin (0.25 %) and EDTA (0.02 %).

The experiments on myeloma and neuroblastoma cells were performed in collaboration with Drs H. Jernberg Wiklund and F. Hedborg, University of Uppsala, Uppsala, Sweden. The experiments with human uterine leiomyoma cells are carried out in collaboration with Drs. K. Gemzell-Danielsson and O. Danielsson, Karolinska Institutet, Stockholm, Sweden.

Methods

Assay of cell growth and survival

Cell proliferation kit II (Roche Inc.) is based on colorimetric change of the yellow tetrazolium salt XTT in orange formazan dye by the respiratory chain of viable cells (Roehm, NW, *et al.*, *J Immunol Methods* 142:257-265, 1991). Cells seeded at a concentration of 5000/well in 100 µl medium in a 96-well plate were treated with different drugs in the given concentration. After 24 or 48 h the cells were incubated, according to the manufacturer's protocol, with XTT labelling mixture. After 4 h the formazan dye is quantified using a scanning multiwell spectrophotometer with a 495-nm filter. The absorbance is directly correlated with number of viable cells. The standard absorbance curve was drawn by means of untreated cells seeded at a concentration of from 1000 to 10 000 cells/ well with an increasing rate of 1000 cells/ well. All standards and experiments were performed in triplicates.

Immunoprecipitation and determination of protein content

The isolated cells were lyzed in 10 ml ice-cold PBSTDS containing protease inhibitors (Carlberg, M., *et al.*, *J Biol Chem* 271:17453-17462, 1996). 50 µl protein A or G agarose was added in 1 ml sample and incubated for 15 min at 4°C on an orbital shaker. After centrifugation for 10 min at 10,000 r/min at 4°C the supernatant was saved. The protein content was determined by a dye-binding assay with a reagent purchased from Bio-Rad. Bovine serum albumin was used as a standard. 15 µl Protein G Plus agarose and 5 µl anti-IGF-1R were added. After 3 h incubation at 4°C on an orbital shaker the precipitate was collected by pulse centrifuga-

tion in a micro centrifuge at 14,000xg for 10 s. The supernatant was discarded and the pellet was washed 3 times with PBSTDS.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were solved in a 2x-sample buffer containing Laemmli buffer and 0.5 % methanol and boiled for 5 min at 96°C. Samples were separated by SDS-PAGE with a 4 % stacking gel and 7.5 % separation gel. Molecular weight markers (Bio Rad, Sweden) were run simultaneously in all experiments.

Western blotting

Following SDS-PAGE the proteins were transferred overnight to nitro-cellulose membranes (Hybond, Amersham, UK) and then blocked for 1 h at room temperature in a solution of 4 % skimmed milk powder and 0.02 % Tween 20 in PBS, pH 7.5. Incubations with the primary antibodies were performed for 1 h at room temperature, followed by 3 washes with PBS with Tween and incubation with the second antibody for 1 h room temperature. After another 3 washes the membranes were incubated with Streptavidin-labelled horseradish peroxidase for 30 min and then detected using Amersham ECL system (Amersham, UK). The films were scanned by Fluor-S (BioRad).

Experiment 1: Dose-response effects of picropodophyllin on viability of human myeloma, neuroblastoma, glioblastoma, and lung cancer cells

The myeloma cell lines EJM, Karpas and RPMI8226, the human neuroblastoma cells SH-SY5Y, the human glioblastoma cells (U-87MG), and the human lung cancer cells (A549) were proven to express the IGF-1R. This was assayed by Western blotting analysis (see experimental). The 6 cell lines were seeded in 96-well plates (medium volume in a well was 100 µl), in medium supplemented with fetal calf serum. After 24 h picropodophyllin was added at different concentrations for 48 h. Cell viability was then assayed (see above). IC_{50} values for each inhibitor and cell lines are

shown below (Table 1). The results are based on 3 different experiments.

Table 1.

IC_{50} (nM) for viability of myeloma, neuroblastoma, glioblastoma and lung cancer cell lines.

Cell line	IC_{50} nM
EJM	190
Karpas	485
RPMI8226	100
SH-SY5Y	50
U-87MG	180
A549	100

The results demonstrate that picropodophyllin has a strong cytotoxic effect on human myeloma, neuroblastoma, glioblastoma and lung cancer cells.

Experiment 2. Dose-response effects of deoxypodophyllotoxin on viability of human myeloma, neuroblastoma, glioblastoma, and lung cancer cells

The myeloma cell lines EJM, Karpas and RPMI8226, the human neuroblastoma cells SH-SY5Y and the human glioblastoma cells (U-87MG), and the human lung cancer cells (A549) were proven to express the IGF-1R. This was assayed by Western blotting analysis (see experimental). The 6 cell lines were seeded in 96-well plates (medium volume in a well was 100 µl), in medium supplemented with fetal calf serum. After 24 h deoxypodophyllotoxin was added at different concentrations for 48 h. Cell viability was then assayed (see above). IC_{50} values for each inhibitor and cell lines are shown below (Table 2). The results are based on 3 different experiments.

Table 2.

IC_{50} (nM) for viability of myeloma, neuroblastoma, glioblastoma and lung cancer cell lines.

Cell line	IC_{50} nM
EJM	7
Karpas	5
RPMI8226	<5
SH-SY5Y	5
U-87MG	100
A549	10

The results demonstrate that deoxypodophyllotoxin has a strong cytotoxic effect on human myeloma, neuroblastoma, glioblastoma and lung cancer cells.

Experiment 3. Effects of picropodophyllin and deoxypodophyllotoxin on cell proliferation of cultured human uterine leiomyoma cells

Isolated and cultured human uterine leiomyoma cells (hULC) are grown in 24-well plates (20.000-40.000 cells/well). The studies on effects of picropodophyllin and deoxypodophyllotoxin on growth and survival of the hULC are performed essentially as described in Experiment 1. In addition, cell proliferation is assessed by measuring (^{3}H)thymidine incorporation into DNA (DNA synthesis) and (^{3}H)leucine incorporation into proteins (protein synthesis). In the former case, the cells (20.000-40.000 cells/well) are grown in 24-well plates and incubated for 24 h with the addition of 1 µCi/ml (^{3}H)thymidine and of IGF-1 (nM-µM concentrations; alone or present in fetal bovine serum) with and without picropodophyllin or deoxypodophyllotoxin at different concentrations (0-1.0 µM). The cells are then washed with F12-medium and DNA is precipitated with 5 % ice cold trichloroacetic acid(TCA). DNA is solubilized in 0.1 M KOH and 500 µl of the solution in each well is added to scintillation liquid and the radioactivity determined in a liquid scintillation counter. In the latter case, cells are incubated for

24 h as described above, but without (^{3}H)thymidine. Instead (^{3}H)leucine is added to reach a concentration of 1 µCi/ml, but only for the last 90 minutes of the incubation. The cells are then rinsed with cold phosphate buffered saline (pH=7.4) and proteins are precipitated in ice-cold TCA. The proteins are solubilized in a solution containing: 5 % sodium dodecyl sulphate, 20 mM Na_2CO_3 and 2 mM EDTA. Radioactivity is determined by liquid scintillation counting. The results on DNA and protein synthesis in hULC are presented as % of control cells, i.e. those incubated without picropodophyllin or deoxypodophyllotoxin.

Experiment 4. Determination of plasma levels of picropodophyllin

A method for determination of levels of picropodophyllin (and deoxypodophyllotoxin) in plasma/serum from mice and humans has been developed in order to correlate plasma levels of the compounds with biological responses in vivo.

Briefly, a solution containing the internal standard ^{14}C-cortisone (about 10,000 cpm) is taken to a tube and the solvent is evaporated under a gentle stream of N_2. Serum/plasma (0.2 - 1.0 ml) is then added and the internal standard is redissolved using an ultrasonic bath and incubation at 37°C for 15 minutes. The sample is diluted twice with water (>0.5 ml)and then passed through a small column of octadecylsilane (ODS) bonded silica (Sep-Pak C_{18} cartridge; washed with 5 ml each of methanol and water prior to use). Following a wash with 2 ml of water and 3 ml each of 20 % aqueous acetone and water, removal of excess of water from the column with a gentle stream of N_2, and a final wash with 3 ml of hexane, picropodophyllin is eluted with 3 ml of ethylacetate/hexane (3:1, v./v.). To this eluate is then added 0.5 ml of 0.2 M aqueous sodium phosphate buffer, pH 7.4. The mixture is shaken and the aqueous phase frozen. The organic phase is taken to another tube and the solvent is evaporated under a gentle stream of

N_2. The sample is then dissolved in 50 % aqueous methanol and after an aliquot has been taken for radioactivity determination, the sample is analysed by reversed-phase HPLC using a column packed with ODS silica (LiChrospher; 100 RP-18 , 5µm; Merck) and a mobile phase of water / tetrahydrofuran / methanol, 360:125:15 (by volume)(or a mobile phase consisting of 55 % aqueous methanol). As an alternative analytical method following evaporation of the solvent picropodophyllin may be trimethylsilylated using 0.2 ml of a mixture of trimethylchlorosilane / hexamethyldisilazane / pyridine, 1:2:3 (by volume) for 30 min at 50°C. The reagent mixture is then evaporated by N_2, the sample is dissolved in hexane and picropodophyllin is analysed by gas chromatography-mass spectrometry.

Experiment 5. Plasma levels of picropodophyllin in mice after a single dose given intraperitoneally

Using the method described in Experiment 4, plasma levels of picropodophyllin in mice could be measured following intraperioneal injections of the compound. Mice (normal and nude) were injected intraperitoneally with different doses of picropodophyllin and were sacrificed after 2, 4, 8 or 24 h. The blood was collected and serum isolated and frozen at -20 °C until analyzed. Table 3 shows the results of these studies. From these data the half-life time of picropodophyllin in plasma of mice could be estimated to be about 1-2 hours (after the initial 2-4 hours).

Table 3.

Concentration of picropodophyllin (PPP) in plasma from mice after a single dose given as an intraperitoneal injection

Time after i.p. injection	Plasma concentration of PPP (μM) after a dose of		
	9 mg/kg	13 mg/kg	25 mg/kg
2 h	>5.0	>5.0	>5.0
4 h	1.19	-	-
8 h	0.14	0.51	0.99
24 h	<0.005	-	-

The results show that a dose of 9 mg/kg will not be sufficient to keep the plasma level of picropodophyllin in the mice above 0.05 μM for 12 h, whereas a dose between 13 and 25 mg/kg should be enough.

Experiment 6. Dose-response effect of picropodophyllin on mice with myeloma

Mice with spread myeloma (5T33 MM) were either untreated or treated with 2 different doses of picropodophyllin. One group was treated with 7 mg/kg/12h and one with 20 mg/kg/12h and the doses were administered intraperitoneally twice daily. The untreated control group was only injected intraperitoneally with the vehicle (totally per day: 40 μl DMSO/vegetable oil, 20:1). For comparison, one group were healthy mice that did not receive any injections. Each group included 9-10 mice. The results after 3 weeks of treatment are shown in Table 4.

Table 4.

The effects of different doses of picropodophyllin (PPP) on mice with myeloma after 3 weeks of treatment.

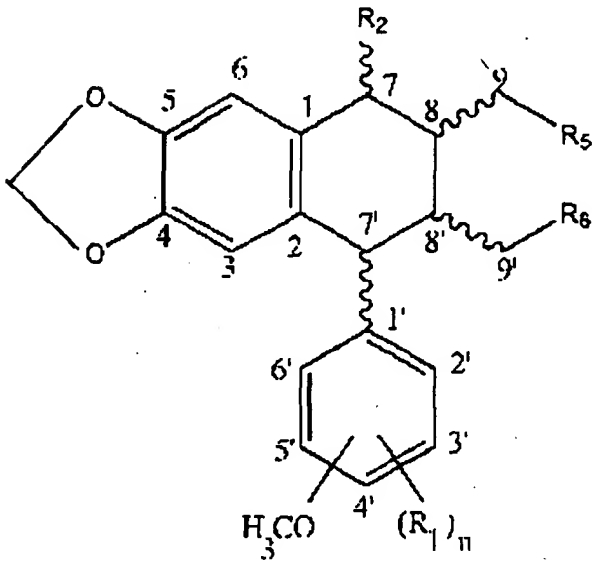
Animal group	Myeloma cells in bone marrow (% of total)	Paraprotein[a] in plasma (g/dl)
Healthy mice	0	0
Control mice with myeloma	72	30
Mice treated with PPP: 7 mg/kg/12h	78	22
Mice treated with PPP:20 mg/kg/12h	16	3

[a]The abnormal paraprotein is formed by myeloma cells and is secreted into the blood stream where it can serve as a marker of tumour mass.

The results show that PPP in a dose of 20 mg/kg/12h has a strong cytotoxic effect on myeloma cells in vivo. With this dose the plasma level of PPP is above 0.05 µM throughout the day. In contrast, PPP in a dose of 7 mg/kg/12h has little or no effect on myeloma cells in vivo. In this case, the plasma level of PPP will drop to below 0.05 µM and may even become undetectable (<0.005 µM) before the next injection with PPP.

CLAIMS

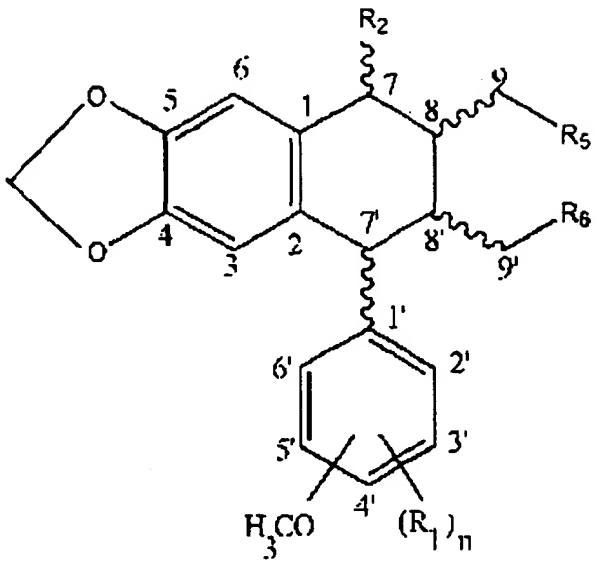
1. Use of a compound of the formula I



I

wherein R_1, which can be the same or different, is OH or OCH_3, n is 0, 1 or 2, R_2 is H, OH, O, NOH, $NOCH_3$, OCH_3, OCOH, OCH_2CH_3, $OCOCH_3$, and R_5 and R_6, which can be the same or different, are H, OH, O, OCH_3, $OOCH_3$, OCOCH, OCH_2CH_3, $OOCH_2CH_3$, or $OCOCH_3$, or together an ether or a lactone, and which optionally contains a double bond $\Delta^{7(8)}$ or $\Delta^{8(8')}$, as a specific inhibitor of the insulin-like growth factor-1 receptor for the preparation of a medicament for treatment of the malignant diseses neuroblastoma, myeloma, glioblastoma and lung cancer.

2. Use of a compound of the formula I

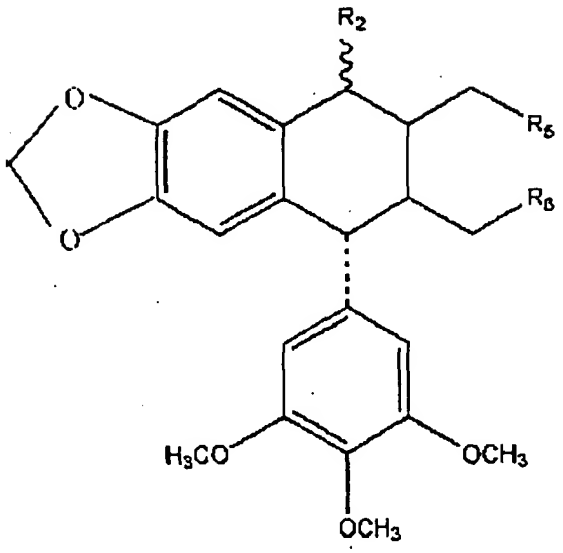


I

wherein R_1, which can be the same or different, is OH or OCH_3, n is 0, 1 or 2, R_2 is H, OH, O, NOH, $NOCH_3$, OCH_3, OCOH, OCH_2CH_3, $OCOCH_3$, and R_5 and R_6, which can be the same or different, are H, OH, O, OCH_3, $OOCH_3$, OCOCH, OCH_2CH_3, $OOCH_2CH_3$, or $OCOCH_3$, or together an ether or a lactone, and which optionally contains a double bond $\Delta^{7(8)}$ or $\Delta^{8(8')}$, as a specific inhibitor of the insulin-like growth factor-1 receptor for the preparation of a medicament for treatment of benign tumours.

3. Use according to claim 2 for the treatment of leiomyoma.

4. Use of according to anyone of claims 1-3 of a compound having the formula II

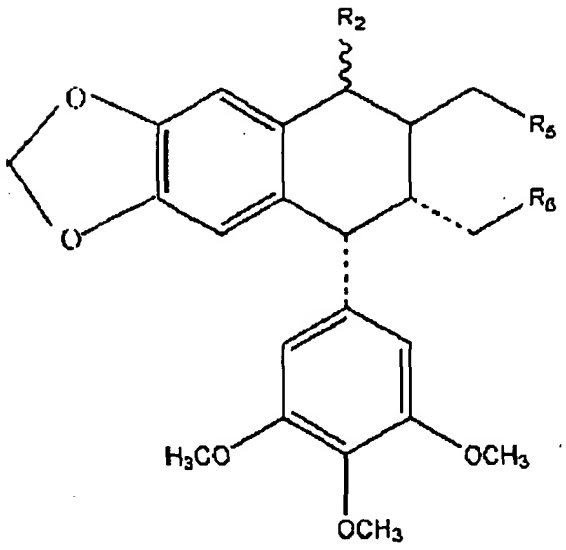


II

wherein R_2 is H, OH, O, NOH, $NOCH_3$, OCH_3, OCOH, OCH_2CH_3, $OCOCH_3$, and R_5 and R_6, in *cis* configuration, can be the same or different, and are H, OH, O, OCH_3, $OOCH_3$, OCOCH, OCH_2CH_3, $OOCH_2CH_3$, or $OCOCH_3$, or together an ether or a lactone, and optionally contains a double bond $\Delta^{7(8)}$ or $\Delta^{8(8')}$.

5. Use according to anyone of claims 1-3 of a compound selected from the group consisting of picropodophyllin, deoxypicropodophyllin, beta-apopicropodophyllin and alpha-apopicropodophyllin.

6. Use according to any one of claims 1-3 of a compound having the formula III



III

wherein R_2 is H, OH, O, NOH, $NOCH_3$, OCH_3, OCOH, OCH_2CH_3, $OCOCH_3$, and R_5 and R_6, in *trans* configuration, can be the same or different, and are H, OH, O, OCH_3, $OOCH_3$, OCOCH, OCH_2CH_3, $OOCH_2CH_3$, or $OCOCH_3$, or together an ether or a lactone, and optionally contains a double bond $\Delta^{7(8)}$.

7. Use according to anyone of claims 1-3 or 6 of a compound selected from group consisting of deoxypodophyllotoxin, epipodophyllotoxin and acetylpodophyllotoxin.

8. Use of a compound according to anyone of claims 1-7 in combination with a cytostaticum.

9. Method of treatment of a neuroblastoma, myeloma, glioblastoma or lung cancer, comprising the steps of administrating a pharma-

ceutical composition, containing a compound having the formula I, as defined in anyone of claims 1, 4-7, in combination with a physiologically acceptable carrier, by constant infusion to a patient suffering from a tumour, monitoring the plasma level of the compound, and adjusting the rate of infusion to keep the plasma level at a concentration of 0.05-5.0 μM, for a period of time being sufficient for the tumour to be retarded or to disappear.

10. Method of treatment of a benign tumour, comprising the steps of administrating a pharmaceutical composition, containing a compound having the formula I, as defined in anyone of claims 2-7, in combination with a physiologically acceptable carrier, by constant infusion to a patient suffering from a tumour, monitoring the plasma level of the compound, and adjusting the rate of infusion to keep the plasma level at a concentration of 0.05-5.0 μM, for a period of time being sufficient for the tumour to be retarded or to disappear.

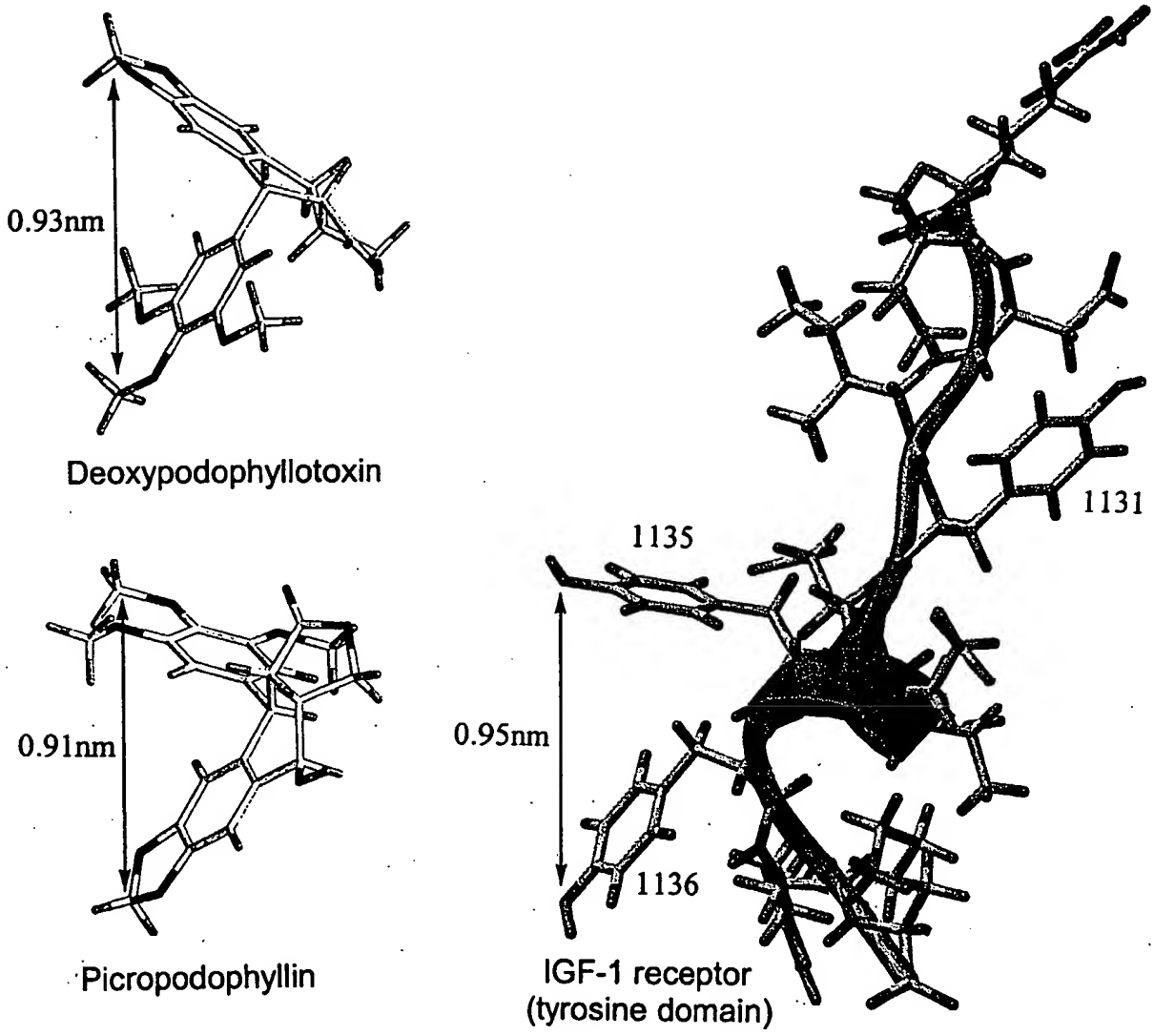
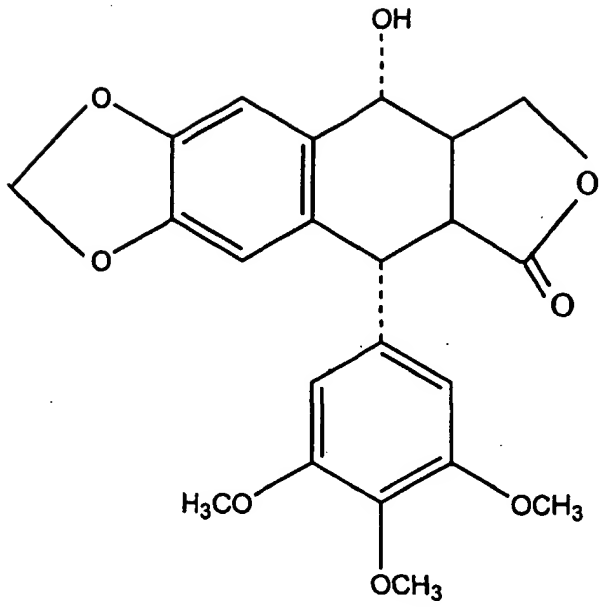
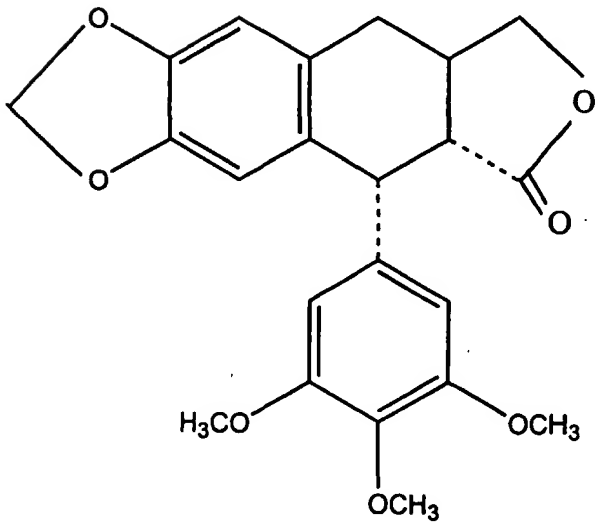

0.93nm
Deoxypodophyllotoxin
0.91nm
Picropodophyllin
1135
1131
0.95nm
1136
IGF-1 receptor
(tyrosine domain)

FIG. 1



Picropodophyllin



Deoxypodophyllotoxin

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2003/002012

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07D 493/06, A61K 31/365, A61P 35/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07D, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHEM. ABS DATA, WPI DATA, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 02102805 A1 (KAROLINSKA INNOVATIONS AB), 27 December 2002 (27.12.2002) --	1-8
X	J. Pharm. Belg., Volume 51, no. 1, J.-C. Doré et al: "Approche par analyse multivariée des relations structure-activités antitumorale et antivirale dans la série de la podophyllotoxine", page 998 - page 3256, see page 14 -- --------	1-8

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 March 2004	09 -03- 2004
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf/EÖ Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2003/002012

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9-10
because they relate to subject matter not required to be searched by this Authority, namely:

see next sheet

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/SE 2003/002012

Box No. IV Text of the abstract (Continuation of item 5 of the first sheet)

Claims 9-10 relate to methods of treatment of the human or animal body by surgery or by therapy or diagnostic methods practised on the human or animal body (PCT Rule 39.1(iv)). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds or compositions.

WO	02102805	A1	27/12/2002	AU	9446001	A 15/04/2002
				CA	2424931	A 11/04/2002
				EP	1325035	A 09/07/2003
				SE	0102168	D 00/00/0000
				WO	02102804	A 27/12/2002